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Implementation of a molecular diagnostic method of citrus black spot (*Phyllosticta citricarpa*) from a single lesion on fruit

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ABSTRACT

It is essential to have a fast and accurate method of diagnosis of the *Phyllosticta citricarpa* fungus in citrus fruits because this pathogen is quarantined for several international markets. For this purpose, an automated DNA extraction methodology was combined from an individual lesion of citrus black spot with the real-time PCR (qPCR) technique. Using this technique, it was possible to distinguish between *P. citricarpa* and the citrus endophytic fungus *Guignardia mangiferae*. The applied qPCR technique allowed the specific detection of *P. citricarpa* as no cross-reaction was observed with any other citrus pathogens or related *Phyllosticta* species. Reliable results were obtained since no false positives were detected. The qPCR with the TaqMan probe showed to be more sensitive compared to the different conventional PCRs analyzed. The real-time PCR proved to be viable, reproducible, specific, and highly sensitive for the detection of *P. citricarpa* under the conditions tested. This diagnostic tool has been proven to be reliable for analyzing citrus samples within certification programs for exporting fruits to markets where *P. citricarpa* is considered quarantine.

Key words: quarantine disease, lemon, detection, real-time PCR.

RESUMEN

Implementación de un método de diagnóstico molecular de la mancha negra de los cítricos (*Phyllosticta citricarpa*) a partir de una única lesión en frutos

Es fundamental disponer de un método rápido y preciso de diagnóstico del hongo *Phyllosticta citricarpa* en frutos cítricos debido a que este patógeno es cuarentenario para varios mercados internacionales. Para esto, se combinó una metodología automatizada de extracción de ADN, a partir de una lesión individual de mancha negra de los cítricos, con la técnica de PCR en tiempo real (qPCR). Mediante esta técnica fue posible distinguir entre *P. citricarpa* y el hongo endófito de los cítricos *Guignardia mangiferae*. La técnica de PCR en tiempo real permitió la detección específica de *P. citricarpa*, y no se observó reacción cruzada con otros patógenos de cítricos ni especies relacionadas de *Phyllosticta*. Se obtuvieron resultados fiables, no se detectaron falsos positivos. La qPCR con sonda TaqMan demostró ser más sensible en comparación con las diferentes PCR convencionales analizadas. La qPCR demostró ser viable, reproducible, específica y altamente sensible para la detección de *P. citricarpa*, en las condiciones evaluadas. Esta herramienta de diagnóstico demostró ser confiable para analizar muestras de cítricos dentro de programas de certificación para la exportación de frutas a mercados donde *P. citricarpa* se considera cuarentenaria.

Palabras clave: enfermedad cuarentenaria, limón, detección, PCR en tiempo real.

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INTRODUCTION

Citrus black spot (CBS) is one of the most important diseases in lemon production in Tucumán province because it is considered quarantine in the European Union (where the disease has not yet been reported), one of the main markets where Argentina fresh citrus fruit is exported. The causal agent is fungus *Phyllosticta citricarpa* (McAlpine) Van der Aa (Van der Aa, 1973) (*Guignardia citricarpa* Kiely) (Kiely, 1948). CBS was first described in Australia in 1895 (Sutton and Waterston, 1966), and has subsequently been detected in Africa, Asia and America (EPPO/CABI, 1997; CABI/EPPO, 1998; CABI, 2011).

All varieties of sweet orange (*Citrus sinensis*) and species such as lemon (*C. lemon*), grapefruit (*C. paradisi*) and mandarin (*C. reticulata*) are susceptible to this pathogen.

This fungus has two types of reproduction, a sexual or teleomorphic state *Guignardia citricarpa* (ascocarps with ascospores) and an asexual or anamorphic state *Phyllosticta citricarpa* (pycnidia with conidia). The main damages caused by this pathogen are spots or lesions on the fruits that affect commercial quality. These fruit lesions exhibit a high variability and can be classified into four main types: hard spot, freckle spot, false melanose and virulent spot. These symptoms can evolve, making it common to find lesions that correspond to intermediate states between aforementioned symptoms.

In Brazil, two other types of symptoms have been reported infrequently in orange, lacey spot (Goes, 2001) which is a variant of false melanose and cracked spot which are slightly raised craked superficial lesions (Goes *et al.*, 2000).

The symptoms of CBS widely described in the literature are easy to recognize visually, but when the reproductive structures (pycnidia) of *P. citricarpa* are absent, direct morphological identification of the causal agent is not possible. There are symptoms that closely resemble by *P. citricarpa*, which are actually associated with other biotic or abiotic causes. Some of the CBS-like lesions commonly seen on citrus are caused by other biotic agents, such as anthracnose (*Colletotrichum gloeosporioides*), melanose (*Diaporthe citri*), greasy spot (*Mycosphaerella citri*) and septoria spot (*Septoria citri*). In addition, other symptoms were reported on citrus fruits like red margin lesions on Marsh grapefruit and minute spots on grapefruit, lemon, and lime fruits associated with *P. capitalensis* (Baayen *et al.*, 2002; Fogliata *et al.*, 2004; 2006; Meyer *et al.*, 2006). Wulandari *et al.* (2009) reported a new species of *Phyllosticta* causing the tanned spot in pummelo (*Citrus maxima*) in Asia called *Phyllosticta citriasiana*.

Since the symptoms caused by *P. citricarpa* in citrus fruits are similar to those caused by other pathogens, a reliable diagnosis can only be made using molecular methods (Kotzé, 2000; Snowdon, 1990; NIMF, 2016). It is very difficult to diagnose the CBS especially in lesions without pycnidia, which can lead to incorrect visual identification. Currently, the diagnosis of this disease requires high precision techniques equivalent as specific molecular techniques for this pathogen, in combination with a high quality DNA extraction. Molecular techniques based on the polymerase chain reaction (PCR) allow a rapid and specific diagnosis of *Phyllosticta citricarpa* (Bonants *et al.*, 2003; Meyer *et al.*, 2006; Peres *et al.*, 2007). van Gent-Pelzer *et al.* (2007) developed a new ultra-sensitive and quantitative real-time PCR (qPCR) diagnostic system with a TaqMan probe, for the detection of *P. citricarpa*, reducing the risks of contamination and also allowing to distinguish it from other non-quarantine *Phyllosticta* species.

The optimization of this system is highly important given the quarantine nature of this pathogen. A misdiagnosis can have detrimental effects on exports, including both false negative result leading to the shipment of fruit infected with CBS, and false positive result that prevent the export of disease-free fruit.

Exporting citrus fruits is key for the sustainability of citrus activity in Argentina. Maintaining the participation in current markets and opening new markets requires phytosanitary support to meet the demands of importing countries.

The objectives of this work were to evaluate the efficacy of a molecular diagnostic methodology for CBS that combines automated DNA extraction with the qPCR technique, and to compare its sensitivity with other available PCR techniques, in order to implement a fast, sensitive system and specifically aimed at the diagnosis of CBS from a single fruit lesion.

MATERIALS AND METHODS

Plant and fungal material

Sweet orange (*Citrus sinensis*), lemon (*C. limon*) and mandarin (*C. reticulata*) fruits with different CBS symptoms such as hard spot, freckle spot and virulent spot were collected in the provinces of Tucumán, Salta and Jujuy, Argentina (Table 1). Fruits with lesions of different sizes (between 0.5 mm and 4.0 mm in diameter) with both absence and presence of pycnidia were selected.

Fruits without disease symptoms (apparently healthy) were also used. Pieces of tissue without symptoms obtained from fruits with CBS symptons and lesions of citrus canker (*Xanthomonas citri* pv. *citri*), melanose (*Phomopsis citri*), citrus scab (*Elsinoë* sp.), red spot (*Guignardia mangiferae*), black mottling (*Guignardia mangiferae*), brown rot (*Phytophthora* sp.), and Diplodia stem-end rot (*Lasiodiplodia theobromae*) of citrus fruits were used as negative controls (Table 2). All fruits were disinfected with pure ethanol. Such different lesions were extracted from the fruit (5 mm diameter discs) by removing and discarding as much surrounding albedo as possible.

In addition, the specificity of the real-time PCR (qPCR) technique was validated by using mycelia of the Phyllosticta citricarpa (T34) as positive control. *Guignardia mangiferae*, *Colletrotrichum* spp., *Galactomyces citri-aurantii*, *Penicillium* spp., *Elsinoë* spp., *Lasiodiplodia theobromae*, *Phomopsis citri* and *Fusarium oxysporum* were used as negative controls. Strains were grown on potato dextrose agar (PDA) for seven days at 25°C.

DNA extraction

Two different commercial DNA extraction kits

Table 1. Fruit samples per host and province used in this study, according to the size and type of citrus black spot lesions with and without pycnidia, EEAOC Phytopathology Laboratory.

Citrus black spot		Number of samples per host and per province					
Type of symptoms	Smaller-larger diameter (mm)	*Citrus sinensis*			*Citrus limon*		*Citrus reticulata*
		Tucumán	Salta	Jujuy	Tucumán	Salta	Jujuy
Hard spot with pycnidia	≤1	1	19	14	1	2	-
	1-2	-	21	6	4	2	-
	2-3	-	4	-	-	-	2
Hard spot without pycnidia	≤1	-	12	-	-	4	-
	1-2	-	-	1	1	4	4
	2-3	-	-	-	-	-	2
Freckle spot with pycnidia	≤1	1	-	4	3	-	-
	1-2	-	-	6	5	-	-
	2-3	-	-	1	-	-	-
Freckle spot without pycnidia	≤1	-	-	-	3	2	-
	1-2	-	-	-	1	1	-
Virulent spot with pycnidia	1-2	-	-	-	1	-	-
	2-3	-	-	1	-	-	-
	3-4	-	1	-	1	-	-

Table 2. List of vegetal material and citrus pathogens used in this study, EEAOC Phytopathology Laboratory.

Host	Identification of the sample	Location, Province	Vegetal material/citrus pathogens
C. limon	MN-126	San Andrés, Tucumán	Brown rot (*Phytophthora* sp.)
	MN-127		Diplodia stem-end rot (*Lasiodiplodia theobromae*)
	MN-128		Citrus scab (*Elsinoë* sp.)
	MN-129		Black speckled (*Guignardia mangiferae*)
	MN-130		Melanose (*Phomopsis citri*)
	MN-131		Citrus canker (*Xanthomonas citri* pv. *citri*)
	MN-132		Red spot (*Guignardia mangiferae*)
	MN-147	Las Talitas, Tucumán	Healthy tissue, diseased fruit of plant with CBS
C. reticulata	MN-145	La Ramada de Abajo, Tucumán	Healthy tissue, healthy fruit of plant without CBS

were tested to determine the efficiency of *P. citricarpa* DNA extraction from a single CBS fruit lesion.

Discs containing the lesion to be tested were placed in 2 ml tubes with three stainless steel beads, containing 125 µl of homogenization buffer (0.02 M phosphate buffered saline, 0.5% Tween 20, 2.0% polyvinylpyrrolidone, and 0.2% bovine serum albumin). Homogenization was carried out at 5000 rpm with a cycle of 80 s in homogenizer (Precellys 24, Bertín Technologies). The DNA extraction kits were PureLink™ Plant Total DNA Purification Kit, Invitrogen (kit 1) and Wizard Genomic DNA Purification Kit, Promega (kit 2). These methods were performed according the manufacturer's instructions with some modifications.

For DNA extraction from fungal material, 100 mg of mycelium were placed in 2 ml tubes with six ceramic beads and homogenized at 5500 rpm with two cycles of 10 s following the Raeder and Broda (1985) extraction protocol modified by the Phytopathology Laboratory of the EEAOC. The quality and quantity of each extracted DNA sample was verified spectrophotometrically by reading the absorbance at 260 nm and 280 nm, using a spectrophotometer instrument (GeneQuant Pro). The overall DNA yield was determined.

Real-time PCR was used to compare the Ct (threshold cycle) values of the extracted DNA with commercial kits.

qPCR optimization

GcF1 (5'-GGTGATGGAAGGGAGGCCT-3') and GcR1 (5'-GCAACATGGTAGATACACAAGGGT-3') primers and TaqMan probe GcP1 (6-FAM/5'-AAAAAGCCGCCCGACCTACCTTCA-3'/TAMRA), as published by van Gent-Pelzer, *et al.* (2007) were used. For the optimization of the qPCR TaqMan probe, the reaction mix consisted of the use of 1x Premix Ex Taq (Takara) testing different concentrations of the GcF1/GcR1 primers (100, 130, 150, 170, 190, 210, 230, 250, 270 and 290 nM) and GcP1 probe (50, 60, 70, 80, 90, 100, 110 and 120 nM), with the addition of 1 µl of the T34 DNA (100 ng/µl) and filled in with nuclease-free water to a final volume of 25 µl. All reactions were performed in duplicate, and each run contained three negative controls, two No Template

Control (NTC) amplifications and *G. mangiferae* DNA. The cycling conditions were: hot start for 10 min at 95ºC and 40 amplification cycles in 2-steps (15 s 95ºC; 1 min 60ºC). The qPCR was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad).

Calibration curve for real time quantitative PCR validation

The construction of the calibration curve was carried out from 100 ng/µl of DNA of the T34 strain (standard positive control). Ten-fold serial dilutions from $4x10^6$ fg/µl to 0.004 fg/µl ($1x10^8$ to 0.1 fg/reaction) were prepared in triplicate using nuclease-free water and calculate PCR reaction efficiencies.

DNA from other citrus pathogenic fungi were used to test the specificity of the technique. Amplification curves to determine the Ct were analyzed using BioRad iCycler software version 3.0.6070.

Efficiency of the qPCR reaction was calculated using the calibration curve.

Evaluation of real-time quantitative PCR for the detection of *P. citricarpa* from a single fruit lesion with black spot

The detection of *P. citricarpa* was performed using DNA samples extracted from single citrus black spot lesion (Table 1). The qPCR conditions were described above. DNA extracted from T34 strain was used as positive control and DNA of the *G. mangiferae* and two NTC were included as negative controls. All experiments were repeated at least once and most of them several times with consistent results. Ct values of <37.00 indicated the presence of *P. citricarpa* DNA.

Comparison of the sensitivity of molecular techniques used for the detection of *P. citricarpa*

The sensitivity of conventional PCR with GCN/GCMR (Peres *et al.*, 2007) and GCF3/GCR7 (Bonants *et al.*, 2003) primers pairs was compared with quantitative real time PCR with specific TaqMan GcP1 probe and GcF1/GcR1 primers (van Gent-Pelzer *et al.*, 2007).

The term Limit of Detection (LOD) was defined as the lowest concentration of genomic DNA detectable with qPCR was used (Seurinck *et al.*, 2005). LOD was determined with 1 µl ten-fold serial dilutions from $1x10^8$ at 0.1 fg/reaction from DNA of T34. Conventional PCR amplifications were carried out in a total reaction volume of 20 µl. PCR mixtures contained buffer (1.25x), dNTP each (25 mM), $MgCl_2$ (1.875 mM), Taq polymerase (4U) (Invitrogen) and 400 nM for the GCN/GCMR primer pair and 390 nM for GCF3/GCR7. The amplifications were carried out in a PTC 150 Minicycler (MJ Research) equipment programmed at 95°C for 3 min, followed by 30 cycles at 95ºC for 30 s, annealing at 65ºC for 30 s and at 72ºC for 30 s, and a final extension for 3 min at 72°C. PCR products were separated by electrophoresis on 1.5% D1-LE agarose gel (Biodynamics).

The qPCR reaction was carried out as previously described and the LOD was analyzed using BioRad iCycler software version 3.0.6070.

RESULTS AND DISCUSSION

DNA extraction

The Ct values of 25 DNA samples extracted with kit 2 showed that the simple automated extraction method was efficient for obtaining Ct values between 27.33 and 34.30 (Table 3). All samples were positive with kit 2. In the case of kit 1, 25 DNA samples were also extracted (Table 3), seven of which were positive with Ct values between 27.81 and 36.65, six samples did not amplify (N/A) and the rest of the samples produced high Ct values (37.17 to 39.68).

The small amount portion of tissue obtained from a single lesion is the main drawback of grinding or macerating for DNA extraction. Peres *et al.* (2007) proposed the rapid alkaline lysis DNA extraction procedure to address this difficulty. However, van Gent-Pelzer *et al.* (2007) demonstrated the advantage of working with an automated extraction system over manual grinding (Bonants *et al.*, 2003; Meyer *et al.*, 2006). Mechanical lysis in a closed system is less sensitive to contamination and is a good alternative to manual grinding. In the present work, the system proposed by van Gent-Pelzer *et al.* (2007), with the use Wizard Genomic DNA Purification Kit, Promega (kit 2) allowed obtaining a higher yield of DNA and a greater number of positives samples with lower Ct values. This is, a faster though more expensive DNA extraction process, making it unavailable for routine use in some laboratories.

Real-time PCR optimization and calibration curve for method validation

The methodology proposed by van Gent-Pelzer *et al.* (2007) for molecular diagnosis of CBS was optimized in our laboratory. For the optimization of the qPCR, different concentrations of primers were tested, reducing it to 190 nM, that showed good efficiency and precision in the reaction.

The validation of the qPCR technique, carried out by using the calibration curve, showed that the reaction efficiency was optimal with a value of 108.8% and a curve slope of -3.128. Assay precision was high (R^2= 0.998) at dilutions of the standard positive control based on linear regression analysis (Figure 1). The Ct values obtained for each dilution showed an increase of 2 or 3 points from each other.

The specificity of TaqMan qPCR for *P. citricarpa* was demonstrated by testing against a related *Guignardia* species and seven different citrus pathogens isolated from citrus fruits (*Colletrotrichum* spp., *Galactomyces citri-aurantii*, *Penicillium* spp., *Elsinoë* spp., *Lasiodiplodia theobromae*, *Phomopsis citri* and *Fusarium oxysporum*). No cross-reactions were observed. Fluorescent signal was only observed using the TaqMan qPCR in the case of *P. citricarpa*.

Detection of *P. citricarpa* from single CBS lesion on fruit

All the DNA samples extracted with kit 2 from a single lesion of fruit with CBS gave positive results. The Ct values of the *P. citricarpa* DNA extracts did not showed

Table 3. Comparison of the Ct values with qPCR with TaqMan probe. Twenty five samples of DNA extracted using kit 1 and twenty five DNA extracted with kit 2 of different size CBS lesions with pycnidia. EEAOC Phytopathology Laboratory.

PureLink™ Plant Total DNA Purification Kit, Invitrogen (Kit 1)				Wizard Genomic DNA Purification Kit, Promega (Kit 2)			
Identification of the sample	Type of symptoms with pycnidia	Smaller-larger diameter (mm)	qPCR Ct values	Identification of the sample	Type of symptoms with pycnidia	Smaller-larger diameter (mm)	qPCR Ct values
MN-114	Hard spot	1.2-1.2	27,81	MN-026	Hard spot	1.5-1.5	27.33
MN-113	Hard spot	1.5-1.5	28,01	MN-004	Freckle spot	1.5-1.5	27.61
MN-111	Hard spot	0.5-0.5	28,94	MN-016	Freckle spot	1.0-1.0	27.77
MN-110	Hard spot	0.5-0.8	30,03	MN-005	Freckle spot	1.2-1.2	28.60
MN-021	Freckle spot	1.0-1.2	32,82	MN-001	Freckle spot	1.0-1.0	28.72
MN-080	Hard spot	1.0-1.0	36,22	MN-071	Hard spot	1.0-1.1	29.13
MN-081	Hard spot	1.2-1.2	36,65	MN-014	Freckle spot	1.0-1.0	29.16
MN-083	Hard spot	1.0-1.0	37,17	MN-062	Hard spot	0.7-0.7	30.10
MN-074	Hard spot	1.0-1.0	37,19	MN-012	Freckle spot	1.0-1.0	30.25
MN-085	Hard spot	1.0-1.1	37,45	MN-092	Hard spot	1.0-1.1	30.56
MN-076	Hard spot	1.0-1.8	38,23	MN-060	Hard spot	1.0-1.2	30.69
MN-077	Hard spot	1.0-1.1	38,24	MN-091	Hard spot	1.0-1.0	31.72
MN-088	Hard spot	0.5-0.5	38,44	MN-023	Hard spot	1.0-1.0	32.06
MN-093	Hard spot	1.0-1.0	38,47	MN-033	Hard spot	1.0-1.0	32.66
MN-075	Hard spot	1.2-1.8	38,86	MN-031	Hard spot	1.0-1.0	33.25
MN-066	Hard spot	1.0-1.2	39,02	MN-032	Hard spot	1.0-1.0	33.26
MN-067	Hard spot	0.8-1.0	39,07	MN-034	Hard spot	1.0-1.0	33.39
MN-095	Hard spot	0.8-1.0	39,56	MN-043	Hard spot	1.0-1.0	33.42
MN-082	Hard spot	1.0-1.0	39,68	MN-039	Hard spot	1.0-1.0	33.62
MN-068	Hard spot	1.0-1.0	N/A	MN-024	Hard spot	1.5-1.5	34.02
MN-072	Hard spot	0.7-1.0	N/A	MN-022	Hard spot	1.0-1.2	34.05
MN-073	Hard spot	1.0-1.0	N/A	MN-037	Hard spot	1.0-1.5	34.16
MN-086	Hard spot	1.0-1.5	N/A	MN-040	Hard spot	1.0-1.5	34.16
MN-089	Hard spot	0.7-0.7	N/A	MN-048	Hard spot	1.0-1.0	34.22
MN-094	Hard spot	1.0-1.0	N/A	MN-044	Hard spot	1.0-1.0	34.30

differences in the lesions analyzed with the presence or absence of pycnidia. In addition, no differences were observed in the Ct values depending on the size of the lesions (Table 4). Although the quantification of the pathogen was not carried out in the different samples analyzed, Ct values are indicative of its concentration in the fruit. Almeida (2009) concludes that there could be a relationship between the concentration of the pathogen and the type of symptom manifested. In the present work, no differences were observed between the concentration of the pathogen in the different symptoms analyzed based on the Ct values, although this requires more studies.

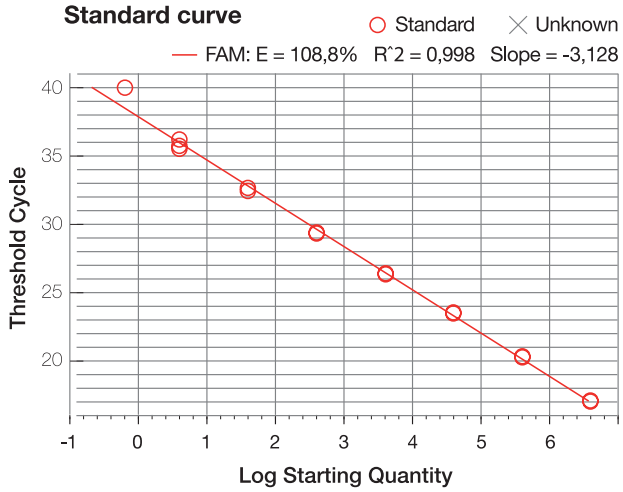

Figure 1. Standard curve derived from real-time PCR with TaqMan probe reactions of a 1:10 dilution series of *Phyllosticta citricarpa* DNA. EEAOC Phytopathology Laboratory.

Detección de *P. citricarpa*: comparison of the sensitivity of different molecular methods

Real-time PCR and conventional PCR methods (Bonants *et al.*, 2003; Peres *et al.*, 2007) revealed

Table 4. Ct values range with TaqMan qPCR in citrus black spot lesions with and without pycnidia and size. EEAOC Phytopathology Laboratory.

		Ct values range *P. citricarpa*
Black spot lesions	With pycnidia	20.79 - 36.02
	Without pycnidia	25.34 - 36.08
Lesion size (mm)	≤1	25.34 - 36.08
	1-2	20.79 - 34.88
	2-3	26.14 - 30.63
	3-4	21.02 - 36.02

differences in the detection sensitivity issue. The LOD for conventional PCR were 10000 fg/reaction with GCF3/GCR7 primers and 1000 fg/reaction with GCN/GCMR primers (Table 5, Figure 2). The LOD for TaqMan qPCR was 100 fg/reaction (Table 5, Figure 3).

Conventional PCR methods (Bonants *et al.*, 2003; Meyer *et al.*, 2006; Peres *et al.*, 2007) for the diagnosis of CBS require a lot of time and the visualization of the amplified product on the agarose gel is difficult to qualify when their levels are low, coinciding with the results reported by van Gent-Pelzer *et al.* (2007). In contrast, Faganello *et al.* (2015) detected *G. citricarpa* by real time PCR in asymptomatic orange leaves, demonstrating the high sensitivity of this technique.

Table 5. Comparison of Limit Of Detection (LOD) of two conventional PCR and real-time PCR using TaqMan probe with serial dilutions of a positive control of CBS. EEAOC Phytopathology Laboratory.

Serial dilutions of positive control	fg/ reaction	TaqMan qPCR (GcF1/GcR1/ GcP1)	PCR (GCN/GC MR)	PCR (GCF3/ GCR7)
T34 Std 4x10^{6}	100000000	+	+	+
T34 Std 4x10^{5}	10000000	+	+	+
T34 Std 4x10^{4}	1000000	+	+	+
T34 Std 4x10^{3}	100000	+	+	+
T34 Std 4x10^{2}	10000	+	+	+
T34 Std 4x10^{1}	1000	+	+	-
T34 Std 4x10^{0}	100	+	-	-
T34 Std 4x10^{-1}	10	-	-	-
T34 Std 4x10^{-2}	1	-	-	-
T34 Std 4x10^{-3}	0.1	-	-	-

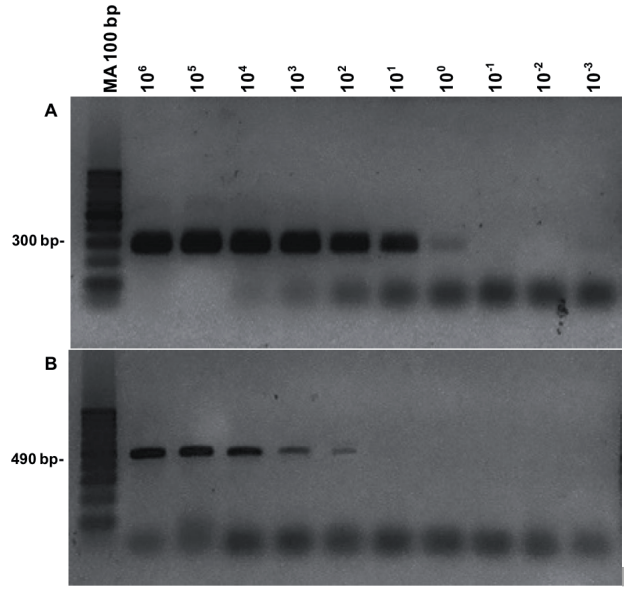

Figure 2. *Phyllosticta citricarpa* detection by conventional PCR. A. primers GCN and GCMR. B. primers GCF3 and GCR7. 1) 100 bp Ladder; 2 to 11) Ten-fold serial dilutions of a DNA from *P. citricarpa* (fungal mycelium). EEAOC Phytopathology Laboratory.

Peres *et al.* (2007) developed ITS region primers that proved to be highly effective to detect and differentiate *G. citricarpa* from *G. mangiferae*. In this study, the sensitivity of these primers was demonstrated, although because of the height of the bands, both pathogens are fall to close, 300 bp and 290 bp, respectively.

CONCLUSIONS

CBS molecular diagnosis system from a single lesion fruit, including an automated DNA extraction followed by qPCR, showed high efficacy and sensibility. TaqMan qPCR technique was from 10 to 100 times more sensitive than conventional PCR. This technique proved to be a valuable support tool for citrus diseases diagnostic laboratories dealing with citrus exporting to markets with quarantine restrictions.

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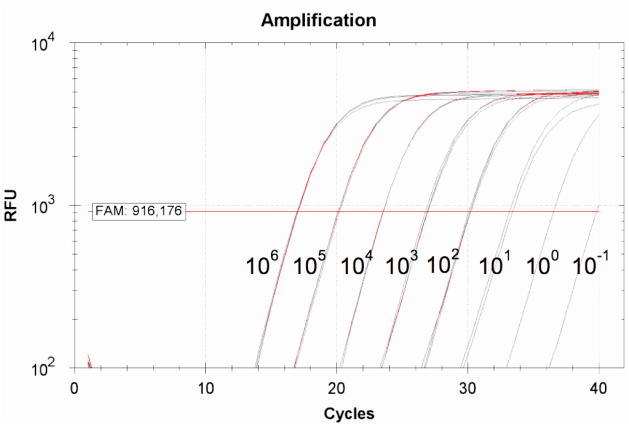

Figure 3. *Phyllosticta citricarpa* detection by TaqMan qPCR with ten-fold serial dilutions of a DNA from *P. citricarpa* (fungal mycelium). EEAOC Phytopathology Laboratory.

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