MOLECULAR DIAGNOSIS OF *Guignardia citricarpa* IN ASYMPTOMATIC SWEET ORANGE TISSUE[1]

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RESUMO – A pinta preta ou mancha preta dos citros, causada pelo fungo *Guignardia citricarpa*, é considerada uma doença quarentenária, que impõe restrições ao transporte de frutas frescas para países da União Europeia. A ocorrência de infecções latentes e o tempo para o diagnóstico por métodos convencionais levam à necessidade de validar protocolos moleculares rápidos, eficientes e reprodutíveis para detecção do patógeno em tecidos assintomáticos. Assim, este trabalho visou detectar *G. citricarpa* em tecidos de frutos sintomáticos e em folhas assintomáticas de laranja Pêra por PCR convencional e por PCR em tempo real. A especificidade e o limite de detecção foram avaliados em amostras de tecidos de lesões em frutos e em folhas assintomáticas. Em folhas assintomáticas a presença do fungo foi detectada em baixas concentrações, nessas condições, a PCR em tempo real demonstrou ser viável, reprodutível e altamente sensível para a detecção do patógeno.

Termos para indexação: Citricultura, diagnose molecular, *Phyllosticta citricarpa*.

DETECÇÃO MOLECULAR DE *Guignardia citricarpa* EM TECIDOS ASSINTOMÁTICOS DE LARANJA-PERA

ABSTRACT – Citrus black spot, a fungal disease caused by the quarantine fungus *Guignardia citricarpa*, restricts the exportation of fresh fruit to countries in the European Union. The occurrence of latent infections and the time required for diagnosis using conventional methods have brought about the need to validate fast, efficient and reproducible molecular techniques to detect the pathogen in asymptomatic tissue. As such, this study aims to detect *G. citricarpa* in the symptomatic fruit and asymptomatic leaf tissue of sweet oranges by conventional and real-time polymerase chain reaction (PCR). Specificity and limit of detection (LOD) were assessed in tissue samples of fruit lesions and asymptomatic leaves. Low concentrations of the fungus were found in asymptomatic leaves. Under these conditions, real-time PCR proved to be viable, reproducible and highly sensitive to detection of the pathogen.

Index terms: Citriculture, molecular diagnosis, *Phyllosticta citricarpa*.

INTRODUCTION

Citrus black spot (CBS), caused by *Guignardia citricarpa* (=*Phyllosticta citricarpa*), is one of the main fungal diseases in citriculture. All varieties of sweet orange (*Citrus sinensis*) and species such as *C. limon*, *C. paradisi*, *C. reticulata* and *C. deliciosa* are susceptible to the pathogen (HU et al., 2014). It was first reported in Australia in 1895 (SUTTON and WATERSTON, 1966) and has since spread to other citrus growing regions, such as southern and central Africa, South America and Asia, becoming a major phytosanitary problem in citrus crops (HINCAPIE et al., 2014; MARTÍNEZ-MINAYA et al., 2015). It was recently identified in the United States, where it is limited to certain areas of Florida (SCHUBERT et al., 2012). Since it is a quarantine disease, CBS limits the exportation of fresh fruit to the European Union (EPPO, 2015).

Symptoms of the disease generally occur on mature fruit after harvest or during transport and storage. However, the infection can remain in a quiescent state and occur in the period between petal fall and fruit maturation. This dormant period

is interrupted by fruit maturation and climate conditions that favor the pathogen. The occurrence of long periods of latent infection demonstrates the importance of early detection, before symptoms emerge (HU et al., 2014).

In addition to being performed after symptom emergence, morphological diagnosis is further hampered by the similarity between *G. citricarpa* lesions and those caused by other pathogens, such as *Alternaria alternata* f. sp. *citri* and *Diaporthe citri*. The existence of an endophytic species (*G. mangiferae*) that is very similar to the pathogen and the occurrence of false negatives when using morphological markers (BAAYEN et al., 2002; WANG et al., 2012; HU et al., 2014) confirm the need for specific and efficient early diagnosis of the disease.

In addition to contributing to plant health, early detection of the pathogen in asymptomatic tissue is essential for the certification of citrus fruits destined for export to countries with stringent phytopathogen legislation. Molecular diagnosis stands out in the early and accurate detection of *G. citricarpa* as a low-cost technique with high specificity, sensitivity and reproducibility. This allows it to meet the high demand for sample processing and comply with the standards of International Plant Protection Organizations for the diagnosis of quarantine diseases, particularly *G. citricarpa* (EFSA, 2014).

Since there are no reports of techniques to detect latent *G. citricarpa* infection, and given the need for efficient and accurate diagnosis of CBR regardless of phenological growth stage, this study aimed to achieve early diagnosis of *G. citricarpa* in asymptomatic sweet orange leaves using conventional and real-time PCR.

MATERIAL AND METHODS

Pathogen isolation and sample preparation

Isolates of *G. citricarpa* were obtained from fruit with typical symptoms of the disease, on 16-year-old sweet orange (*C. sinensis*) plants collected in a commercial orange grove in the municipality of Pirenópolis, Goiás state (GO), Brazil. The pathogen was identified by analyses and morphological indicators and kept in oatmeal agar medium (BAAYEN et al., 2002).

Fruits with citrus black spot lesions were collected from 20 plants, totaling 20 oranges. Of these, ten were washed using detergent and rinsed

with distilled water, and ten were used without being cleaned. The samples consisted of 250 mg tissue fragments from lesions on washed or unwashed fruit, as well as samples from a single lesion (measuring about 2 to 4 mm). All the samples were placed in 2 mL microtubes and stored in a freezer at -20 °C for subsequent DNA extraction.

Concomitantly, asymptomatic leaves were collected from orchards on two properties in the municipalities of Pirenópolis and Inhumas (GO). In Pirenópolis, samples were collected from a 40-hectare orchard of 16-year-old sweet orange plants (*C. sinensis*). Symptoms were observed on mature fruit concentrated in an 8-ha area of the orchard. Sampling was carried out in a 32-ha area that showed no visible symptoms of CBS. In Inhumas, sampling was performed in a 6.5-ha area containing 15-year-old sweet orange trees. No CBS symptoms were observed on the plot during sampling.

Asymptomatic leaves were randomly collected from 20 plants on each property. Eight leaves were collected from each plant at three different heights (lower, middle and upper third), totaling 24 leaves per plant. Two 0.5-cm wide sections were removed from each set of 8 leaves, one from each side of the midrib, and cut into smaller evenly-sized fragments. The working samples consisted of 250 mg of fragments, totaling 60 samples, which were stored at -20 °C until DNA extraction.

DNA extraction

The DNA extraction protocol was modified from the CTAB method and optimized at the Plant Diagnosis Laboratory (LDV) of the National Agricultural Laboratory in Goiás (LANAGRO-GO) (MORELLO, 2000). During the extraction process, reagent blanks (extraction controls) and environmental controls were used to demonstrate the lack of external nuclei acid contamination. In order to rupture the cell wall, 250 mg samples were placed in 2mL microtubes added with 1000 µL of 2% CTAB buffer {p(CTAB) = 20 g L^{-1}, c(NaCl) = 1.4 mol L^{-1}, c(tris) = 0.1mol L^{-1}, c(Na2EDTA) = 0.02 mol L^{-1}; pH 8.0 adjusted with HCl}, previously heated to 65 ºC, and two glass beads were used to break the cell walls. The microtubes were agitated for 1 minute in a TissueLyser II bead mill at a maximum frequency of 30 oscillations per second. At the end of the process 20 µL of proteinase (20 mg mL^{-1}) were added and the microtubes were incubated in a water bath (65 ºC) for one hour, with gentle agitation every ten minutes. The supernatant was transferred to a new microtube and added with 520 µL of a 24:1 (v/v) CIA solution (chloroform: isoamyl alcohol), with manual

inversion for 5 minutes.

For DNA precipitation, the samples were centrifuged at 12,000 g for ten minutes and 600 µL of the supernatant was transferred to nine 1.5 mL microtubes. Next, 300 µL of ammonium acetate (7.5 mol L^{-1}) and 600 µL of cold 96% isopropanol were added. After continuous, gentle manual inversion the solution was centrifuged at 12,000 g for 10 minutes and the supernatant was discarded. The precipitate was washed once with 500 µL of 70 % ethanol, centrifuged again at 12,000 g for ten minutes and resuspended in 50 µL of TE (Tris = 10 mM; EDTA = 1 mM, pH 8.0). The microtubes were then added with 1 µL of RNase A (10 mg mL^{-1}) and incubated at 37°C ± 2 ºC for thirty minutes. The DNA samples were kept in a freezer at -20 °C until quantification.

Specificity analysis and limit of detection of *G. citricarpa* by conventional PCR

The protocol developed by Bonants et al. (2003) was used for specificity and limit of detection (LOD) testing in conventional PCR (EPPO/OEPP, 2009). The reaction conditions were: 0.60 µM of each primer, GcF3 (5'–AAA AAG CCG GAC CTA CCT–3') and GcR7 (5'–TGT CCG GCG GCC AG–3'); 1x buffer (10x); 0.060 mM of dNTp Mix; 1.5 mM of MgCl$_2$; 1 U of Taq DNA polymerase; 100 ng µL^{-1} of DNA; and type I water (nuclease-free) to obtain a final volume of 25 µL. Amplification was performed in a thermocycler (LongGene®: MG96G), with an initial denaturation cycle of 94 ºC for two minutes, thirty 30 s cycles at 94 ºC, 30s at 65 ºC, one minute at 72 ºC, and a final ten-minute cycle at 72 ºC. The amplification products were separated by electrophoresis using agarose gel (1 %), stained in ethidium bromide solution (0.5 mg L-1) and visualized in a transilluminator under ultraviolet light (320 nm).

The specificity of the primers (GcF3 and GcR7) was assessed by amplification reaction of the DNA extracted from *G. citricarpa* lesions on the washed and unwashed oranges, from the single *G. citricarpa* lesion on disinfected and non-disinfected fruit, *G. citricarpa* isolates, and isolates of the fungi *Alternaria* sp., *Fusarium oxysporum* and *F. solani*, the insect *Diaphorina citri*, and the phytobacteria *Candidatus* Liberibacter asiaticus and *Ca*. L. americanus, as well as phytoplasma isolates.

Concentrations of *G. citricarpa* mycelial DNA were used for LOD testing, adjusted from 100 ng µL^{-1}, followed by 4 serial dilutions (10^{n}) with ten replicates each.

Specificity analysis and limit of detection of *G. citricarpa* by real-time PCR

The protocol developed by Van Gent-Pelzer et al. (2007) was used for real-time PCR, applied to specificity and LOD tests (EPPO/OEPP, 2009). The reactions were prepared using 0.25 µM of each primer, GcF1 (5'–GGT GAT GGA AGG GAG GCC T–3') and GcR1 (5'–GCA ACA TGG TAG ATA CAC AAG GGT–3'); 0.1µM of the GcP1 probe (5'–AAA AAG CCG CCC GAC CTA CCT TCA–3'); 1x TaqMan (Ampli Taq Gold®); 100 ng of DNA; and type I water (nuclease-free) to obtain a final reaction volume of 30 µL. The amplifications were carried out in a thermocycler (Eppendorf® Mastercycler ep Gradient), with initial denaturation at 95 ºC for ten minutes, 45 fifteen-second cycles at 95 ºC and one minute at 60 ºC.

To assess analytical specificity by real-time PCR, DNA from the same samples used in conventional PCR was submitted to reaction amplification. For LOD testing, an initial solution of 100 ng µL^{-1} of *G. citricarpa* DNA was submitted to seven serial dilutions with ten replicates.

G. citricarpa detection in asymptomatic leaves

The detection of *G. citricarpa* in asymptomatic leaves was tested by comparing the LOD between conventional and real-time PCR, using the same DNA extraction protocol and the same methodology corresponding to each detection technique.

RESULTS AND DISCUSSION

The DNA extracted from all the samples showed structural integrity, evident in the visualization of genomic DNA in 1 % agarose gel and low polyphenol and polysaccharide concentrations, considering the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios (data not shown). The possibility of high quantity and quality DNA extraction from citrus fruit tissue using the method proposed here, with a view to detecting *G. citricarpa* by conventional and real-time PCR, is more practical, offers greater autonomy and reduces costs 7.5-fold on average in relation to commercial extraction kits (DEMEKE and JENKINS, 2010).

Analyses using specific molecular markers (GcF3 and GcR7) to identify the pathogen by conventional PCR amplified the 490 bp bands of the 16S ribosomal region of the positive controls. There was no amplification for DNA extracted from the other microorganisms or insect species, confirming the specificity of the technique against pathogens or

pests that cause other diseases in citrus plants (Figure 1). As a result of the higher amount of fungal DNA extracted directly from the mycelium, the bands for these samples were more intense than those of the samples from the fruit lesions and the single lesion, a result also observed by Peres et al. (2007).

The amplification products of the 10 replicates of serial dilutions (1:10, $1:10^2$, $1:10^3$ and $1:10^4$) obtained 100 % detection beginning at 100 ng μL^{-1}, 90 % at 10 ng and 70 % at concentrations of 1 and 0.1 ng visualized in agarose gel (1 %). Thus, conventional PCR was suitable for detecting *G. citricarpa* DNA with 90% repeatability of detection at a concentration of 10 ng and 70% at a minimum of 0.01 ng. No amplification was observed at 0.01 ng in agarose gel.

In real-time PCR analysis, the primers and probe used (GcF1 and GcR1/GcP1) were specific for *G. citricarpa*. The amplification products of the 10 replicates of serial dilutions (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$), beginning with 100 ng of *G. citricarpa* mycelial DNA, produced amplifications for all the dilutions via real-time PCR, establishing the LOD with repeatability at a fungal DNA concentration of 10^{-5} ng (10 fg) (Table 1). Real-time PCR was suitable for detecting *G. citricarpa* DNA at a minimum concentration of 232 DNA copies, equivalent to 0.00001 ng of DNA. The number of copies was estimated as proposed by Hu et al. (2014). In addition to validating the in-house technique, these results indicate that the method is robust and more sensitive than conventional PCR, which may allow the phytopathogen to be detected in asymptomatic plant tissue, that is, before symptoms emerge.

For *G. citricarpa* detection using DNA extracted from asymptomatic leaves, conventional PCR was unable to amplify the expected products of the reaction for either the samples from Pirenópolis or Inhumas, totaling 120 samples (Table 2). These results differ from those obtained for the positive controls used in the reactions, which produced an amplicon for the GcF3/GcR7 marker. By contrast, in real-time PCR using the GcF1/GcR1 primers and GcP1 probe, *G. citricarpa* was identified in DNA from asymptomatic orange leaves collected at the two properties studied (Table 2).

Low levels of the fungus were recorded in asymptomatic leaves collected from areas with no CBS symptoms. This may explain the negative result in conventional PCR, which is inefficient at detecting the pathogen under these conditions, since it is less sensitive than real-time PCR. The highest detection frequency was observed in the municipality of Pirenópolis, where the presence of the pathogen on symptomatic fruit was confirmed in a specific plot on the property.

In this study, tests were conducted on asymptomatic leaves without inducing fungal growth prior to DNA extraction and amplification. This differs from research conducted by Meyer et al. (2012), who obtained positive results for *G. citricarpa* detection with conventional PCR, but induced emergence of the fungus on asymptomatic leaves by successively wetting and drying them over a period of four to ten days. Thus, the present study stands out for the specificity, sensitivity and speed of CBS detection prior to symptom emergence using real-time PCR for diagnosis in asymptomatic leaves, highlighting the importance of the technique in certifying the quality of citrus fruits and preserving disease-free areas.

The diagnosis of *G. citricarpa* in an asymptomatic orchard is highly beneficial in its prevention and control. It is important to note that although real-time PCR is highly sensitive, with an LOD of 0.00001 ng of DNA, 100 % detection was not achieved in the test samples, likely because some of the leaves collected showed no quiescent infection in the subcuticular mycelium. However, the detection frequency obtained was highly satisfactory for the identification of *G. citricarpa* in asymptomatic leaves, providing another alternative for preventive detection and diagnosis.

Identifying CBS infection prior to symptom emergence allows the application of more efficient control methods. Moreover, since *G. citricarpa* is a quarantine fungus subject to phytosanitary restrictions, real-time PCR can be used for preventive detection and as an additional option in CBS diagnosis to comply with regulations governing the exportation of fresh fruit to the European Union.

TABLE 1-Number of DNA copies and mean Ct observed for amplification of the specific *Guignardia citricarpa* region by real-time PCR at different DNA concentrations.

DNA (ng µL⁻¹)	Positive/negative samples	Mean Ct	No. of copies*
100	10 / 0	22.18	232×10^7
10	10 / 0	23.00	232×10^6
1	10 / 0	25.70	232×10^5
0.1	10 / 0	28.20	232×10^4
0.01	10 / 0	30.36	232×10^3
0.001	10 / 0	32.28	232×10^2
0.0001	10 / 0	35.79	232×10
0.00001	10 / 0	39.51	232

* Number of copies estimated according to an average molecular weight for a DNA base pair of 4.3×10^{-6} pg (Hu et al., 2014).

TABLE 2- Detection frequency of *Guignardia citricarpa* by conventional and real-time PCR in asymptomatic leaves collected from the upper, middle and lower third of orange trees on two farms in the municipalities of Pirenópolis and Inhumas, Goiás state.

Position	No. of samples	Pirenópolis		Inhumas	
		Conventional PCR (%)	Real-time PCR (%)	Conventional PCR (%)	Real-time PCR (%)
Upper third	20	0	92.5	0	12.5
Middle third	20	0	85.0	0	32.5
Lower third	20	0	77.5	0	35.0
Total/mean	**60**	**0**	**85.0**	**0**	**26.7**
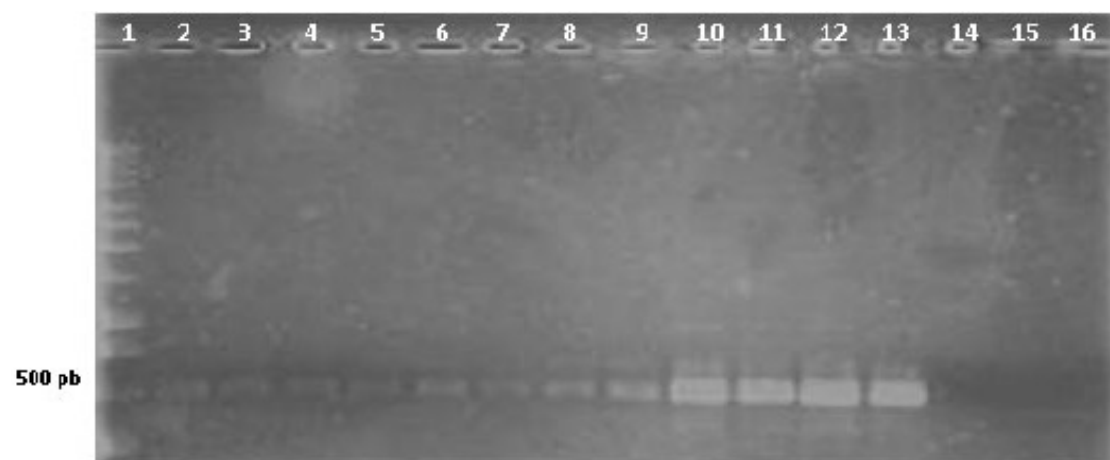
FIGURE 1- *Guignardia citricarpa* detection by conventional PCR (primers GcF3 and GcR7), for specificity testing. 1) 1Kb Ladder; 2 and 3) DNA from a single lesion on washed fruit; 4 and 5) DNA from a single lesion on unwashed fruit; 6 and 7) DNA from lesions on washed fruit; 8 and 9) DNA DNA from lesions on unwashed fruit; 10, 11, 12 and 13) DNA from *Guignardia citricarpa* isolates (mycelium); 14 and 15) DNA from *Alternaria* sp. isolate; 16) Blank.

CONCLUSIONS

Real-time PCR is efficient and applicable in the diagnosis of *G. citricarpa* in asymptomatic orange orchards, showing sensitivity for detection of the pathogen in leaf tissue with no visible symptoms.

Given the proven specificity of the primers used, conventional PCR can be used to identify CBS in symptomatic fruits.

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